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Age-related change in the association between a polymorphism in the *PER3* gene and preferred timing of sleep and waking activities

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Summary

The objective of this study was to investigate the effect of age on the association between preferred timing of sleep and waking activities and a coding-region variable number tandem repeat (VNTR) polymorphism in the clock gene *PER3*. We have previously reported this polymorphism to associate with diurnal preference and delayed sleep phase syndrome (DSPS). Participants (n = 1590; 707 males and 883 females) completed the Horne–Östberg (HÖ) questionnaire for diurnal preference and provided a DNA sample. Overall HÖ scores were plotted against age. The 5% extremes and intermediates were selected for genotyping. Frequencies of the *PER3* 4- and 5-repeat alleles were examined in separate age groups (18–29, 30–39, 40–49 and 50+ years of age). The 4-repeat allele was significantly more frequent in evening types, and the 5-repeat allele more frequent in morning types (Fisher's exact test, P = 0.016). Analysis in the four age groupings revealed that the strength of this association attenuated with age and was significant only in the youngest group (18–29 years). These results extend our previous finding of an association between the *PER3* VNTR and diurnal preference. They also demonstrate that diurnal preference in young people is more closely associated with this polymorphism than it is in other age groups.

Keywords

circadian rhythms; diurnal preference; genetic; polymorphism; sleep; tandem repeat sequences

Introduction

The circadian pacemaker and sleep homeostasis are considered to be the main physiological processes that determine sleep timing. Variation in the timing of sleep and waking activities may be related to variations in key parameters of the circadian timing system, including its sensitivity to light and its endogenous period. The Horne–Östberg (HÖ) questionnaire provides a quantitative measure of diurnal preference (Horne and Östberg, 1976). The HÖ score has been validated by the demonstration that it correlates with the timing of the: (i)

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core body temperature rhythm, and melatonin rhythm as assessed during constant routine protocols (i.e. in the absence of the masking effects of the sleep–wake and light–dark cycle) (Duffy *et al.*, 1999) and (ii) sleep–wake cycle (Kerkhof and Van Dongen, 1996; Taillard *et al.*, 2003). The HÖ questionnaire consists of 19 items relating, for example, to time of day preference for exercise and mental alertness, as well as levels of tiredness upon waking and before going to bed. Internal consistency has been reported to be high ($\alpha = 0.82$). Thirty years later, the original 19-item scale remains the most widely used tool for measuring diurnal preference.

Several parameters affect diurnal preference. Evidence for a genetic contribution has been derived from twin studies. Monozygotic twins are more likely to exhibit shared diurnal preference than dizygotic twins, up to 50% of the variance being estimated to be accounted for by heritability (Vink *et al.*, 2001). Gender also affects diurnal preference, females having been shown to have a higher tendency towards morningness than males (Adan and Natale, 2002; Robilliard *et al.*, 2002; Vink *et al.*, 2001).

Age is another major factor relating to HÖ score. Several reports demonstrate that morningness increases with age (Carrier *et al.*, 1997; Hur *et al.*, 1998; Robilliard *et al.*, 2002; Taillard *et al.*, 2004; Tankova *et al.*, 1994). It has been suggested that this follows an eveningness peak reached at the end of adolescence (Roenneberg *et al.*, 2004). The age-related shift towards higher scores (i.e. morningness) on the HÖ scale is associated with earlier wake times and earlier timing of the endogenous rhythms of core body temperature and melatonin (Duffy *et al.*, 1999). Analysis of the factors underlying these age-related changes has indicated that they are only partially related to changes in parameters of the circadian pacemaker. Age-related changes in sleep need/duration are also thought to contribute considerably to these age-related changes in diurnal preference (Dijk *et al.*, 2000; Duffy *et al.*, 1999). Some studies have developed specific tools to address these age-related changes (Paine *et al.*, 2006) and to assess chronotype accurately from changing patterns of sleep/wake schedules due to social factors (Roenneberg *et al.*, 2003).

Diurnal preference has also been investigated in relation to lifestyle regularity using the Composite Scale of Morningness and Social Rhythm Metric diary (SRM-5) (Monk *et al.*, 2004). Morning types have been reported as having higher scores on the SRM-5, indicating greater daily lifestyle regularity than intermediate or evening types, with evening types having the lowest scores and therefore the least daily regularity (Monk *et al.*, 2004). Eveningness has been linked to increased impulsivity (Caci *et al.*, 2005), which suggests that diurnal preference may have implications for psychiatric evaluations and measurements.

Studies investigating clock gene polymorphisms and circadian phenotype have demonstrated that polymorphisms in the clock gene *PER3* are associated with diurnal preference and may be linked with circadian rhythm disorders such as delayed sleep phase syndrome (DSPS) (Archer *et al.*, 2003; Ebisawa *et al.*, 2001). The coding region of the *PER3* gene contains a variable number tandem repeat (VNTR) which has evolved in primates, with inter- and intra-specific length polymorphism (Jenkins *et al.*, 2005). In humans, we have reported the longer allele (five tandem repeats) to associate with morningness, and the shorter allele (four tandem repeats) with eveningness and DSPS (Archer *et al.*, 2003). This polymorphism does

not appear to show a significant latitudinal cline or balancing selection (Nadkarni *et al.*, 2005), but the same association with diurnal preference (although not with DSPS) has been confirmed in a Brazilian population (Pereira *et al.*, 2005).

The aim of the present study was to explore further the association between the reported *PER3* VNTR polymorphism and diurnal preference in a larger sample. The effect of age on the allele frequencies within populations with extreme morning or evening preference was also examined. The allele frequencies within populations with extreme morning or evening preference were examined in different age groups.

Methods

Participants

Visitors to the London Science Museum participated in the study either in 2001 (n = 484; 217 males and 267 females) (Robilliard *et al.*, 2002), or in 2004 (n = 1106; 490 males and 616 females) and were aged between 18 and 81 years. In the more recent study, in analogy with the previous one, volunteers were recruited from the visitor population at the Science Museum over a 9-week period (September–November 2004) as part of the Live Science exhibition in the Wellcome Wing of the Science Museum. All visitors were invited to participate regardless of country of residence. Multiple members of the same family were excluded from the genotyping part of the study. Written informed consent was obtained from all volunteers after explanation of the project rationale. This study complied with the Declaration of Helsinki, and approval was granted by the University of Surrey Ethics Committee.

Participants completed a computer-based version of the HÖ diurnal preference questionnaire, and received a print-out of their score. They were also asked to provide a buccal swab (MasterAmp Catch-All Sample Collection Swabs; Epicentre Biotechnologies, Madison, WI, USA) for DNA extraction, and to complete a short hard copy questionnaire relating to their usual sleep habits, such as usual bedtimes, sleep latency, number of awakenings etc.

Genotyping

Horne–Östberg scores were plotted against age and a linear regression line was applied to the plot. Those participants who were outside of the 5% cut-off lines parallel to the regression line (i.e. those with the most extreme scores of morningness or eveningness, n = 80 in each group) were selected for genotyping. Participants falling on, or very close to, the regression line were selected as representative intermediate samples (n = 80). DNA was extracted from the selected groups using BuccalAmp DNA Extraction Kits (Epicentre Biotechnologies). Genotyping was performed as described previously (Archer *et al.*, 2003). Allele frequency associations were analysed using Fisher's exact test (two-sided, Instat version 3.01; Graphpad Software, San Diego, CA, USA).

Results

Description of the sample

Morningness, as measured by the HÖ questionnaire, increased with age (linear regression $F = 198.77$; $y = 0.2749x + 42.193$; d.f. = 1; $P < 0.05$). There were no significant differences found between the 2001 and 2004 data sets for age [$t(1,590) = 0.95$, $P > 0.05$] nor HÖ score [$t(1,590) = 0.13$, $P > 0.05$]. Similarly, there was no significant difference between the two data sets for gender ($\chi^2 = 0.85$, d.f. = 1, $P > 0.05$). The combined HÖ frequency distribution was tested for normality using the Kolmogorov–Smirnov test and was found not to satisfy the conditions for assumed normality, exhibiting skewness towards higher HÖ score (skewness = −0.088; kurtosis = −0.78; $P < 0.05$). The extreme morning and evening types ($n = 80$ in each group) were tested for differences in the number of males and females comprising the extreme distributions. The results of the χ^2-test showed that no significant gender differences existed ($\chi^2 = 2.30$, d.f. = 1, $P > 0.05$).

Examined independently, females had a mean HÖ score (±standard deviation) of 52.4 ± 11.9, whilst males had a mean score of 51.4 ± 11.0. Both distributions taken from the entire sample ($n = 1590$) were tested separately for normality, and were found not to conform to a normal distribution, with females showing more skewness towards higher scores (Kolmogorov–Smirnov; skewness = −0.155 for females; −0.074 for males; kurtosis = −0.458 for females; −0.514 for males; $P < 0.05$). The male and female distributions were analysed for differences between the genders with the Mann–Whitney test and found to be not significantly different from each other ($u = 295\,912$; $Z = -1.78$; $P = 0.07$).

Analysis of *PER3* genotype versus HÖ score

The frequencies of the 4- and 5-repeat alleles in the 5-percentile of individuals with extreme morning and evening preference were tested for associations. A significant association between *PER3* VNTR genotype and diurnal preference was found (Fisher's exact $P = 0.016$, odds ratio = 0.53, 95% confidence interval 0.33–0.85). The 4-repeat allele was more strongly associated with evening types (0.75 in evening types; 0.62 in morning types) and the 5-repeat allele with morning types (0.38 in morning types; 0.25 in evening types). Similarly, Fisher's exact test was performed on the intermediate types compared to extreme morning and evening types (4-repeat = 0.63, 5-repeat = 0.37). No significant difference was found between the allele frequencies in morning types and intermediates (Fisher's exact $P = 0.91$, odds ratio = 0.95, 95% confidence interval 0.60–1.49). However, a significant difference was found between intermediate and evening types (Fisher's exact $P = 0.02$, odds ratio = 0.56, 95% confidence interval 0.34–0.90). The observed genotype frequencies for the extreme diurnal preference groups were found to be in Hardy–Weinberg equilibrium (Hardy, 1908). When males and females were analysed separately, the distribution of allele frequencies for each gender was consistent with those found in the combined sample. This suggests that gender did not affect the association between the *PER3* polymorphism and diurnal preference.

The effect of age on the 4-repeat allele frequency was investigated in the 5% extremes (Fig. 1). The plot showed that as age increased, the frequency of the 4-repeat allele

became closer to the population mean of 0.68 (Archer *et al.*, 2003; Nadkarni *et al.*, 2005), decreasing in evening types, and increasing in morning types. Chi-squared analysis revealed an overall significant difference in allele frequency between the extreme morning and evening types when examined across age groups ($\chi^2 = 13.95$, d.f. = 3, $P < 0.001$). Further analysis revealed that there was a clear separation (deviation from the population mean) in phenotypes of younger subjects (18–29 years old). This deviation decreased with increasing age, so that in the 40 to 49-year-old group both extremes had allele frequencies that were indistinguishable from each other and from the combined population mean. In subjects aged 50 and over, an apparent separation was visible again. Fisher's exact test was performed on these data, and a significant difference was found between allele frequencies in the morning and evening types at age 18–29 years only (odds ratio = 0.34; 95% confidence interval = 0.132–0.890; $P = 0.036$). Similarly, when the high versus low scores (based upon the mean allele frequency score) were compared against an age dimension of younger (18–39 years) and older (40+ years) subjects a significant difference was found between these two groups ($\chi^2 = 27.23$, d.f. = 3, $P < 0.001$). No significant association was observed in the other age groups. The age, HÖ score and genotype distribution for the eight sub-groups is shown in Table 1.

Discussion

Data from a large sample of 1590 participants [including $n = 484$ from our previous study (Robilliard *et al.*, 2002)] showed a significant association between diurnal preference and the *PER3* VNTR polymorphism, with the shorter allele (4-repeat) being associated with eveningness and the longer allele (5-repeat) associating with morningness. This is in accordance with our previous report (Archer *et al.*, 2003) and an independent replication in a Brazilian population (Pereira *et al.*, 2005). Females appeared to be more skewed towards morningness than males, as has been reported previously (Adan and Natale, 2002; Robilliard *et al.*, 2002), although the differences between the distributions of males and females were not statistically significant. The trend for increasing morningness with age was also observed within these data.

When different age groups of extreme morning and evening individuals were examined in relation to *PER3* genotype (using allele frequency of the 4-repeat allele) an effect of age on allele frequency was found. There was a significant overall difference between the extreme morning and evening types in terms of their allele frequency across age. More specifically, younger subjects (18–29 years) with extreme diurnal preference displayed a significant deviation from the population mean, indicating that their diurnal preference may be more strongly influenced by their genotype and less influenced by exogenous factors. With increasing age, the allele frequency became closer to the mean population frequency of 0.68. This finding suggests that the diurnal preference of older people, particularly within the 40–49-year age range, may not be as closely linked with genotype but more influenced by social factors such as family responsibilities and daily work routines. Another recently published study also suggested that the original classification criteria of Horne and Östberg (1976) are not useful for the classification of chronotype in middle-aged subjects (Paine *et al.*, 2006). From age 50 years onwards, the allele frequencies of the extreme individuals appeared to exhibit a trend towards separating from the mean once again, although the

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difference was not significant. This observation may suggest that the influence of family and work schedules on the HÖ score begins to weaken, with people in this age group perhaps returning to a lifestyle more in keeping with their endogenous circadian phenotype. Indeed, it has previously been reported that the association between circadian period and entrained circadian phase was stronger in younger than in older subjects (Duffy and Czeisler, 2002), suggesting that these age-related changes in the strength of the associations with diurnal preference can also be observed at the physiological level.

The age-related change in allele frequency between younger (18–39 years of age) and older subjects suggests that the association between the *PER3* polymorphism and diurnal preference is strongest in young people. This finding probably ought to be given consideration when selecting participants for diurnal preference studies with and without a genetic component, and when analysing the results. Differentiation by age group may also be a valuable tool for future genetic association studies of diurnal preference.

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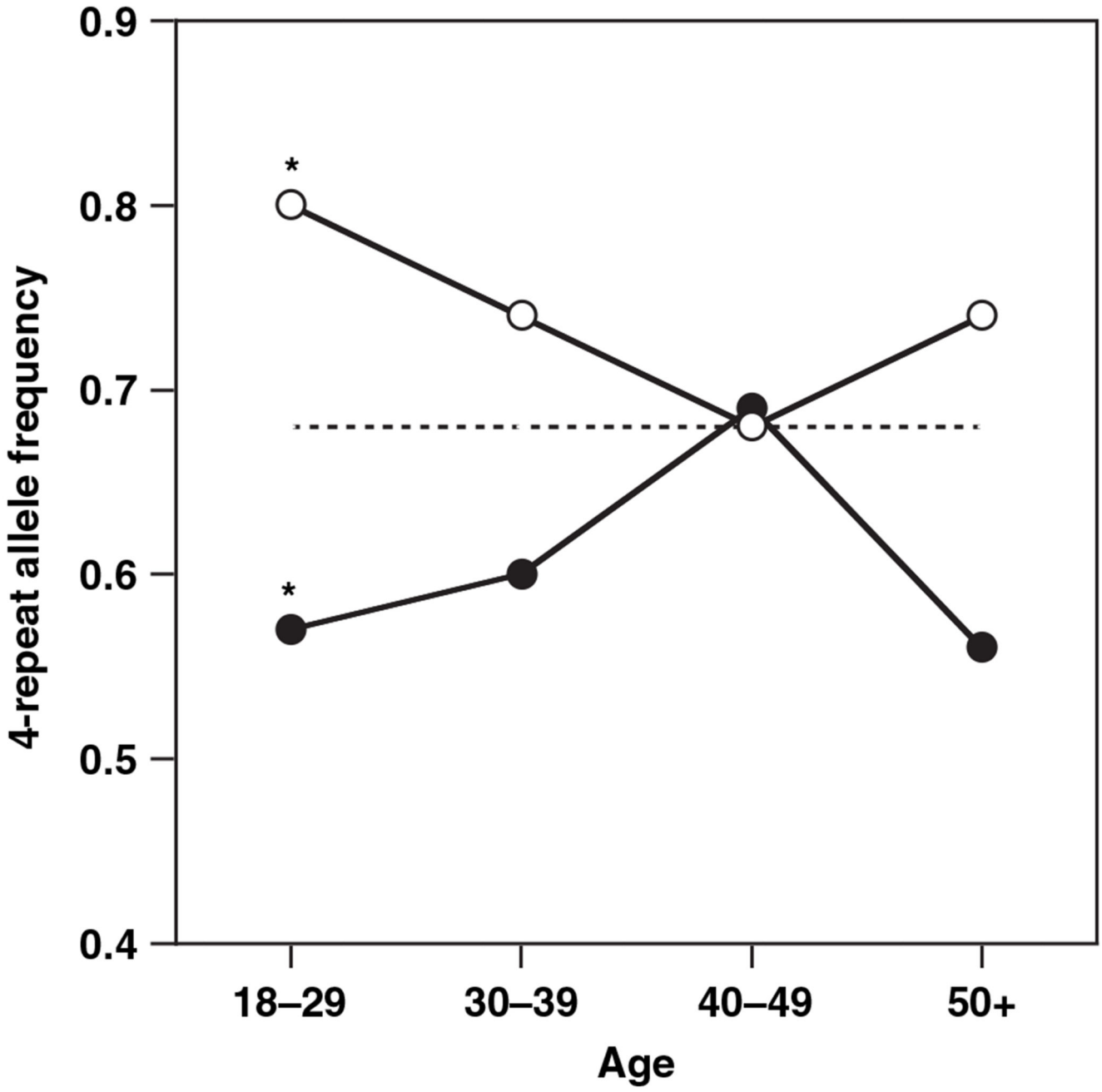

Figure 1.

Frequency of the 4-repeat allele across age groups in extreme morning (filled circles) and evening (open circles) types. The dotted line represents the combined population mean for the 4-repeat allele frequency (0.68). Asterisk denotes a statistically significant association between allele frequency and diurnal preference.

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Table 1

Age, Horne–Östberg (HÖ) score and genotype distributions across age ranges

	Morning types		*Evening types*	
Age range	*HÖ score range*	*Genotype frequency*	*HÖ score range*	*Genotype frequency*
18–29	65–78 (*n* = 21)	4/4 = 0.38	21–29 (*n* = 22)	4/4 = 0.64
		4/5 = 0.38		4/5 = 0.32
		5/5 = 0.24		5/5 = 0.04
30–39	67–83 (*n* = 25)	4/4 = 0.40	21–33 (*n* = 21)	4/4 = 0.57
		4/5 = 0.40		4/5 = 0.33
		5/5 = 0.20		5/5 = 0.10
40–49	69–80 (*n* = 24)	4/4 = 0.46	25–35 (*n* = 11)	4/4 = 0.45
		4/5 = 0.46		4/5 = 0.45
		5/5 = 0.08		5/5 = 0.10
50+	70–78 (*n* = 10)	4/4 = 0.30	25–44 (*n* = 26)	4/4 = 0.58
		4/5 = 0.50		4/5 = 0.34
		5/5 = 0.20		5/5 = 0.08